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14. ABSTRACT This study has rested on two basic hypotheses. The first is that agents given during and (for a short period of time) after resuscitation can favorably influence the response to hemorrhagic shock and injury. The second is that studies of gene response studies, specifically microarray and RT-PCR studies, could be used to assess the response of patients being treated for injuries to such therapeutic agents. To date, both hypotheses have been shown to be true in the animal model. Three therapeutic agents have been identified, to be used during and after resuscitation. Studies are ongoing to validate these hypotheses in patients, including the use of glutamine in patients immediately following resuscitation.					
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Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	6
Reportable Outcomes.....	15
Conclusion.....	18
References.....	19
Appendices.....	N/A
1. Précis of presentation to be given at ESPEN 2010, 24-28 August, 2010	
2. Manuscript on use of omega-3 fatty acids in shock, accepted for publication, Journal of Parenteral and Enteral Nutrition, 2010	
3. Abstract for American Society of Parenteral and Enteral Nutrition, February 8-12, 2010.	

INTRODUCTION

Shock is a leading cause of death among American soldiers wounded in battle. If an injury is not immediately lethal, most deaths result from hemorrhagic shock or from its late sequelae, septic shock and multiple organ failure. The critical time in shock appears to be the period during which the patient is being resuscitated. Resuscitation is associated with a massive activation of the inflammatory reaction, producing immunosuppression, and rendering the patient vulnerable to sepsis and its sequelae. The **goal** of this research program is to develop new treatments for hemorrhagic shock which can be administered before or during initial resuscitation. These treatments are intended to be applied by front-line responders on the battlefield (and first responders in civilian life) as well as by fixed facilities, such as a Forward Surgical Team or a Combat Support Hospital. These agents must be non-toxic and have a very broad therapeutic ratio, so that they can be given safely to injured soldiers, and must be easy to administer under combat conditions. In previous work, the xanthine oxidase inhibitor allopurinol was found beneficial in a shock model (1). In other work, using an animal model of hemorrhagic shock (2), glutamine, glutamine-alanine dipeptide, and arginine have shown efficacy. (3,4) Studies with crocetin have also shown efficacy (5), but this agent is not available in a form which can be administered to patients. Others have advocated the use of DHEA, but studies in our laboratories have failed to show a useful effect (6). Studies with omega-3 fatty acids have shown promise, but are still at an early stage.

The **method** of the research is to measure the response of the genome to hemorrhagic shock using microarray studies. The effect of these agents in patients will be compared to their effect in the animal model, to assess whether they will be effective in the clinical setting.

BODY

We have proposed to develop a pharmacologic strategy, with the aim of reducing the intensity of the post-shock and post-injury inflammatory response. This strategy should be based on one (or more) agent(s) which are safe and can be given early in resuscitation, even by first responders and before resuscitation has begun.

The identification of such agents, which can be given at the beginning of resuscitation, or even before fluid resuscitation is begun, has been the subject of a great deal of research. In our own laboratories, during previous studies using several experimental animal models of hemorrhagic shock, we have identified several compounds which have shown therapeutic effectiveness, specifically crocetin, glutamine, and glutamine-alanine dipeptide. (1-9) There is convincing experimental evidence that these agents enhance cellular recovery following shock, and, importantly, that they significantly improve survival. These agents all appear to have two beneficial effects. First, they appear to reduce mitochondrial damage, which is perhaps the mechanism for improved recovery of tissue ATP levels. Reduced mitochondrial damage has been shown by decreased levels of cytoplasmic cytochrome c and by decreased activity of caspases, notably caspase 3 and 7, and by decreased apoptosis. Second, they appear to suppress the

cytokine response by inhibiting the activation of the transcription factor NF- κ B. The activation of NF- κ B is dependent on the proteasome, a large cellular proteolytic complex that, among other functions, has a regulatory role in the inflammatory process. In parallel investigations, agents that inhibit proteasome activity have also been shown to suppress cytokine production in response to inflammatory stimuli. (10-18) Ongoing research has established similarities between cytokine release induced by sepsis and that induced by hemorrhagic shock. It is becoming increasingly apparent that these similarities may reflect similar changes in the proteasome, a line of research which has been pursued by our laboratories, and which touches on the present investigation in significant aspects.

Issues Encountered

Progress towards the four research objectives, while significant, has been slower than would be desired. The microarray studies in the animal models have been slowed by the relocation of the laboratory of the collaborating investigators to new quarters in a newly-built research building. Authorization for initial human studies was not obtained until June, 2007, and it proved difficult to find suitable subjects. One of the key individuals, Dr. R. Yang, who carried out the animal studies, returned to China somewhat abruptly and unexpectedly. New personnel are being recruited. On the other hand, the PI (Dr. Van Way) was named to a research professorship (Sosland Chair of Trauma Research) effective July 1, 2008, and has been able to devote considerably more effort to the project. Recruitment of patients has been expanded, and the therapeutic arm of the study (Objective #4) is about to begin. However, authorization for this phase has also been time-consuming. Final IRB approval should be obtained by the end of June, 2010, and the study has been approved by the DOD review. Three of the four research objectives have been completed, and the fourth is on track to begin enrolling patients as soon as final IRB approval has been granted.

KEY RESEARCH ACCOMPLISHMENTS

Research objective #1: Establish and validate the micro-array studies, using the animal model. *Deliverable:* A panel of genes that are reproducibly altered in white blood cells and in liver and muscle by shock and resuscitation.

1. These preliminary studies have been carried out, and presentation and/or publications are being prepared. (See list of publications, below). Collaboration continues with the laboratory of Dr. Peter Smith, Kansas University Medical Center, Kansas City, Kansas. This facility carries out micro-array analysis of both animal and human genomes.
2. To briefly summarize the work in this area, the four experimental groups noted below were studied. Even in the sham group, genomic response was quite marked. The microarray analysis used 31,099 probes. These data, which are typical of the entire data set, indicates that even the sham surgical procedure was a strong stimulant to the gene response in white blood cells. The overall pattern of the response to untreated shock and to shock with either form of resuscitation was qualitatively similar. This data supports previous work by many others indicating that so-called hemorrhagic shock procedures are, in fact, a combination of trauma and shock. As noted below, it is quite feasible to identify genes which are differentially responsive to sham, shock, and shock with resuscitation. Also refer to the pathway analysis, below.

Experimental group	Genes upregulated	Genes downregulated
Sham at 30 minutes vs sham baseline	4993	7178
Shock at 30 minutes vs shock baseline	4758	7579
Shock + LR at 30 minutes vs shock + LR baseline	5554	7510
Shock + LR + arginine at 30 minutes vs baseline	6143	6325

3. As noted in the previous-year report, a list of signature genes has been defined. The methodology for identifying these genes was as follows. All of the gene responses were uploaded into a web-based system maintained by Ingenuity Pathways Analysis (Ingenuity Systems, www.ingenuity.com ; see also below, #8). This system combines data analysis with an extensive genome knowledge base. Genes which showed a strong up-regulation or down-regulation (at least fivefold) to shock, as compares with sham controls, were selected. They were considered as possible signature genes if they showed a difference in their response to shock and to one of the two treatment arms, either fluid resuscitation (shock + LR) or fluid resuscitation plus arginine (shock + arginine). The genes meeting these criteria numbered 390. Selection among these was then carried out by eliminating genes which appeared to be irrelevant to the inflammatory reaction, such as oncogenes. Some genes were selected because they appeared to be related to the known components of the inflammatory reaction (cytokines). Several genes were placed on the list because their response in this shock model suggested that they

would be good markers of the response to the surgical preparation and to shock (B3GALNT, F3, GRID2, GRM5). The final list of 20 “signature” genes (below) are to be considered representative of genes which react strongly to the shock preparation, and which appear to be modified by treatment.

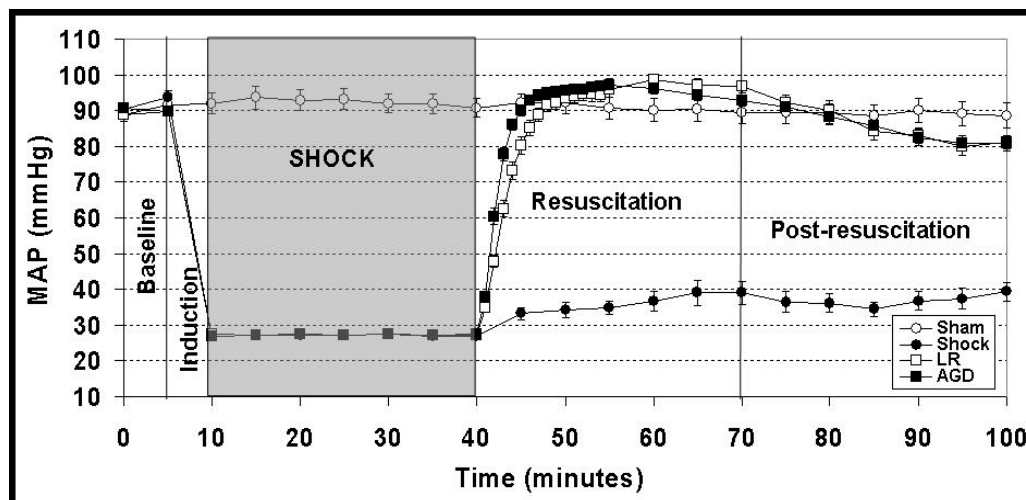
Signature Genes

<i>Gene</i>	<i>Protein and function</i>
ALOX5	Arachidonate 5-lipoxygenase (cytoplasmic enzyme)
B3GALNT1	Beta 1,3-N-acetyl/galactosaminyltransferase 2, membrane-bound galactotransferase enzyme
CXCL3	Chemokine (C-X-C motif) ligand 3, chemokine, cytokine
CXCL11	Chemokine (C-X-C motif) ligand 11, chemokine, cytokine
F3	Coagulation factor III, membrane receptor
GRID2	Glutamate receptor, ionotropic, delta 2 (ion channel)
GRM5	Glutamate membrane receptor
HSPA1B	Heat shock 70kDa protein 1b
HSPBAP	HSPB (heat shock 27kDa) associated protein 1, binds HSPB1
IL1A	Interleukin-1, alpha, cytokine
IL1R2	Interleukin 1 receptor, type II, membrane receptor
MSR1	Macrophage scavenger receptor 1, membrane receptor
NOS2A	Nitric oxide synthase 2A (inducible), cytoplasmic enzyme
NLGN1	Neurologin 1, membrane receptor (neuronal)
NPY1R	Neuropeptide Y receptor Y1, membrane receptor (G-protein coupled)
NR4A3	Nuclear receptor (subfamily 4, group A, member 3), steroid-thyroid hormone receptor superfamily
PLAUR	Plasminogen activator, urokinase receptor, membrane receptor
RGS1	Regulator of G-protein signaling 1, membrane regulatory protein
ST3GAL3	ST3 beta-galactoside alpha-2,3-sialyltransferase 3, membrane glycosyltransferase enzyme
TNF	Tumor necrosis factor (TNF superfamily, member 2), cytokine

4. More detailed analysis of metabolic and signaling pathways has been carried out, using pathway analysis. The data has been presented in last year’s annual report.
5. To facilitate future work in this area, development continues on an assay system incorporating RT-PCR, using a microplate system. (TaqMan Gene Expression Assay, Applied Biosystems, Foster City, CA). In this system, 96-well plates will be made up, to incorporate all or some of the above genes, each replicated four times. There will be 8 genes used as standards (“housekeeping” genes), which will be genes un-affected by the surgical preparation, shock, or treatment. The response of the signature genes will be referenced to these standard genes. This system will allow four replications of a single sample to be determined. It is

anticipated that this system will provide validation of the microarray studies, but more importantly, will enable future studies to be done with considerably better accuracy and reproducibility.

6. The figure illustrates the model which has been developed for use in future experiments. It has been modified somewhat from the model used in the above experiments, in which animals were monitored for four hours following the end of the shock period. The particular series of experiments illustrated used the dipeptide alanine-glutamine dipeptide, which is a glutamine equivalent used clinically in Europe and elsewhere (Dipeptivin). Sprague-Dawley male rats, 300-350 grams, were anesthetized with Isoflurane. Cannulation of the femoral artery and vein were carried out. (19) Hemorrhagic shock was induced by withdrawing blood (0.5 ml/min) to lower the blood pressure to 25-30 mm, and maintaining it at that level for 30 minutes. Resuscitation was by lactated Ringer's solution, 21 ml/kg over 30 minutes. Animals were sacrificed 1 hour after the end of the shock period, and blood withdrawn. This model differs in important respects from the model used in the arginine studies described above, in that only one sampling will be done, at 1 hour, rather than at 30 minutes and 4 hours. The original model showed fairly clearly that later sampling showed similar patterns of gene activation as the early sampling, but appeared more attenuated.



Research objective #2: Define the effect on gene expression of the agents which will be used clinically, using the animal model. *Deliverable:* The effect of the agents upon the level of expression of the signature genes.

1. The agents which have been proposed to be used clinically agents are allopurinol, glutamine, and arginine. Glutamine is an amino acid which has been approved for use in humans, Arginine is also an amino acid which strongly ameliorates the inflammatory reaction, and has shown benefit in the rat model. Allopurinol is available in an intravenous form, and will be used in the further studies. This drug was shown to provide survival benefit in the canine shock model 20 years ago, by the PI and others (1). It appears to act by inhibiting xanthine oxidase, and hence

lowering the production of free radicals during reperfusion. Survival studies with each of these agents have been carried out. Current studies were done in the rat model for glutamine (20) and arginine. Previous work by the PI has shown survival benefit for allopurinol in both the canine shock model and the rat model. Previous studies were also done on alanine-glutamine dipeptide (21), crocetin (22), and dehydroepiandrosterone (DHEA).

Glutamine studies are presented in this table.

Survival with or without Glutamine in Resuscitation

	LR	Glutamine
Rat weight (gram)	295.83±3.03	293.00±3.84
Cannulating Time (minutes)	6.50±0.29	6.50±0.23
Bleeding (ml)	9.22±0.41	9.05±0.58
*Survival > 24 hours	4/12	11/12

Shock at MAP = 22±4 mmHg, 90 minutes. Resuscitated with Ringer's Lactate (LR, 21ml/kg) with/without glutamine (630mg/kg) over 30 minutes. Data expressed as mean ± SEM. *P< 0.05 (LR vs Glutamine).

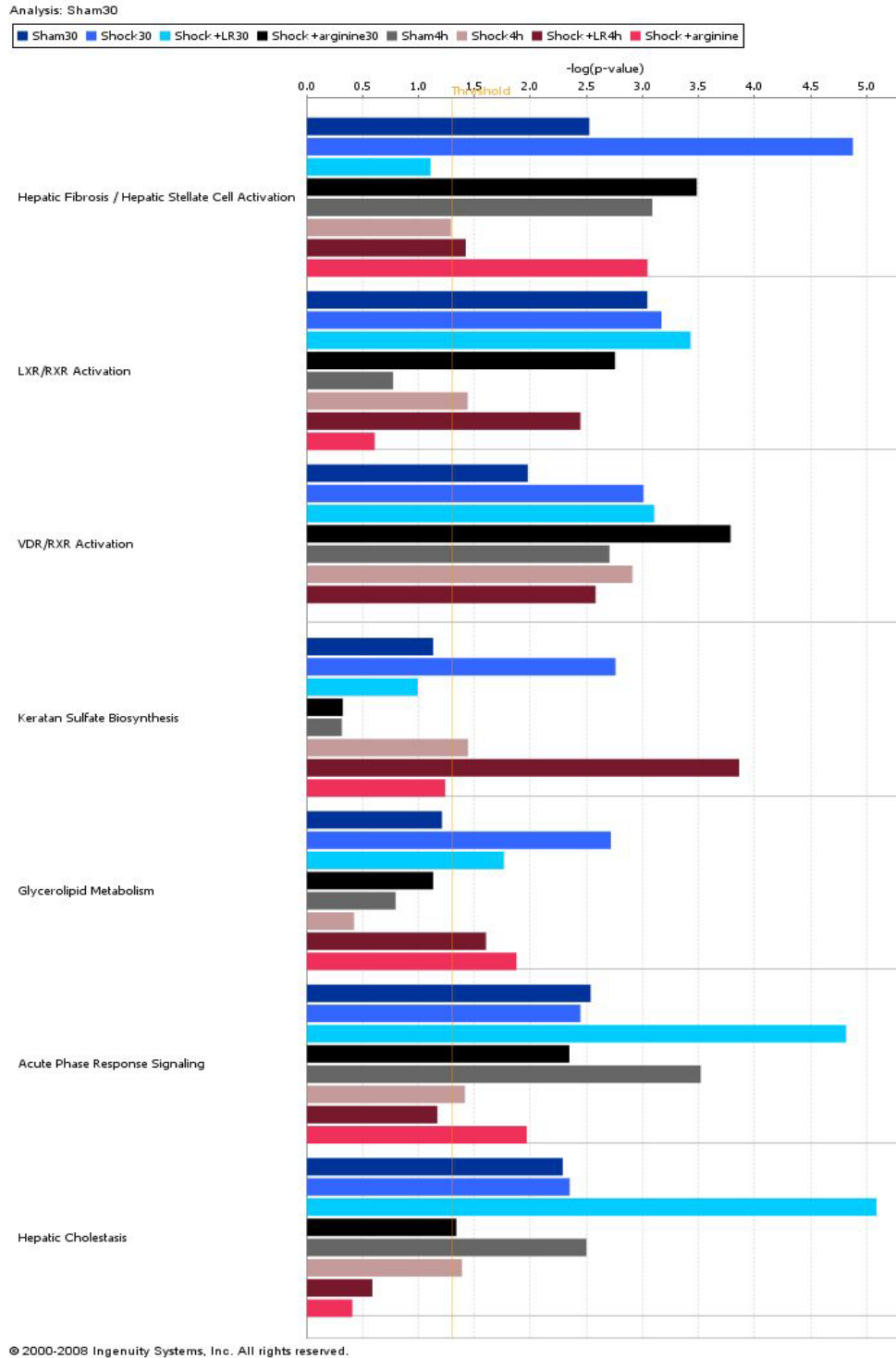
Arginine studies are presented here. In this model, arginine was nearly as effective as glutamine.

Survival with or without Arginine in Resuscitation

	LR	Arginine
Rat weight (gram)	286.70±4.03	294.40±4.81
Cannulating Time (minutes)	6.35±0.13	6.80±0.17
Bleeding (ml)	9.32±0.31	10.06±0.35
*Survival < 24 hours	11/20	4/20
Survival > 72 hours	6/20	12/20

Shock at MAP = 20±4 mmHg, 90 minutes. Resuscitated with Ringer's Lactate (LR, 21ml/kg) with/without arginine (300mg/kg) over 30 minutes. Data expressed as mean ± SEM. *P< 0.05 (LR vs Arginine).

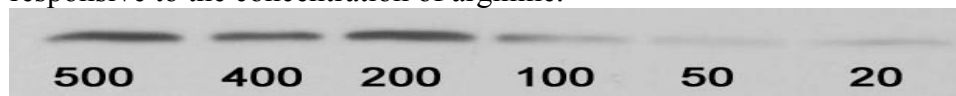
- The genome data from the shock experiment described in Objective #1 has been analyzed further, specifically in the analysis of the two shock groups treated with and without arginine. The objective will be to determine the differential effect of adding arginine. Also shown here is the relative activation of pathways as measured by up-regulation of genes in those pathways. Relative activation of genes is shown in the seven pathways most affected by shock. Within each group, activation is compared with the baseline values for that group. For each pathway, the first four bar graphs represent sham 30 minutes, shock 30 minutes, shock resuscitated with LR at 30 minutes, and shock resuscitated with LR and arginine at 30 minutes. The second four bar graphs represent the same experimental groups at 4 hours. Most of the pathways, with some exceptions, show an attenuated response at four hours.



- A cell-culture based model has been developed, using a standard macrophage cell line (RAW 264.7). This model is based on exposing cultured macrophages to ischemia in a sealed chamber, and then re-exposing them to levels of oxygen found in room air (21%) Reducing the level of oxygen to 1.5% from the usual 21% produces a significant hypoxic insult. This corresponds to a partial pressure of oxygen of 10 mm

Hg. Measuring the effect of therapeutic agents on hypoxia-challenged cell lines, will provide further evaluation of their possible therapeutic effectiveness. Several significant preliminary observations have been made.

- a. Cells grown in hypoxia grow at a rate about 15% less than cells in normoxia for 24 hours, and their growth slows markedly thereafter as compared with normoxic controls.
- b. Adenosine triphosphate (ATP) levels within the cells were measured using a luciferase assay. Data indicates that ATP in cells left chronically ischemic for up to 24 hours have the same ATP content per cell as cells grown in normoxia,
- c. Hypoxia produces a differential response to iNOS. Ischemic cells show an increase in mRNA for iNOS (by RT-PCR), but no increase in nitric oxide activity, and no increase in protein content (by Western blot).
- d. Studies strongly suggest that stress-induced modifications of host responses are associated with a change in the proteolytic subunits of the multifunctional cellular proteasome. This was confirmed in the hypoxic macrophage model, in which the chymotrypsin-like activity, but not the trypsin-like or post-glutamate activities, was increased in hypoxia. Further, this change in activity was found to be responsive to changes in arginine concentrations.
- e. In analyzing the protein subunits which comprise the proteasome, we found over-expression of LMP2 protease as compared with constitutively-expressed X protease. Moreover, this over-expression was responsive to changes in arginine concentrations. In the Western blot below, bands reflect relative levels of LMP2 protease and the numbers indicate the arginine concentration in culture media. Levels of LMP2, for example, appears to be highly responsive to the concentration of arginine.



This appears to be dependent upon which agent is employed, as LMP2 levels are not responsive to varying the concentration of glutamine. Allopurinol has not yet been tested.

- f. The proteasome is central to many of the cell's immune functions, and to its response to inflammatory stimuli, including MHC-directed antigen presentation and regulation of cytokine production. Arginine concentration appears to be important in regulation of proteasomal activity. This underscores the concept which we expect to develop to maturity in further research, namely that changes in levels of arginine and/or glutamine at the level of the cell can profoundly affect the inflammatory response at the cellular level through changes in the proteasome.
- g. Grant funding to carry this line of research further is being sought. In addition, expansion is planned to the study of intestinal epithelial cells (Caco2) and to hepatic cells (HEP-4).

Research objective #3: Assess gene expression in patients treated with current initial resuscitation protocols. Deliverable: Coordinated with objective #1, A panel of genes

that are reproducibly altered in white blood cells and in liver and muscle by shock and resuscitation.

1. The experimental protocol was approved by the Institutional Review Board of the University of Missouri – Kansas City. It was reviewed and approved by the Human Research Protection Office (HRPO), Office of Research Protections (ORP), U.S. Army Medical Research and Materiel Command (USAMRMC).
2. Under this protocol, blood samples have been taken from patients arriving in the trauma center who are injured severely enough to exhibit blood loss or signs of hypovolemia. Blood samples have been collected within one hour following resuscitation, again at 24 hours, and at 7 days or time of discharge from hospital, whichever comes first. These samples are then processed to isolate the white blood cells and extract the RNA, and then submitted for microarray analysis. A total of 15 patients have completed the study.
3. Several conclusions can be drawn. First, the genomic response is maximal at the first hour following resuscitation, with progressively less response at 24 hours and at 7 days or discharge day. Second, there is broad response across the genome, but large responses (four-fold up or down) is confined to a relatively smaller group of genes. Third, many of the genes involved in inflammation are highly up-regulated following trauma.
4. There have been a number of limitations to this study. First, the requirement for obtaining informed consent for the study has limited enrollment to patients with only mild to moderate trauma and blood loss. A further limitation is that only those patients whose mental faculties are intact can participate in the consent process. It has not been possible to enroll patients with severe shock, because there is no time for an adequate informed consent before the patient must be taken to the operating room. Typical time from emergency department admission to operating room is around 10 minutes in severely injured patients. Second, sample processing has been difficult, because the method initially used for white blood cell isolation was time-consuming and technically difficult. Since many patients arrive at night or on weekends, it has been difficult to carry out the processing. However, a commercially available white blood cell isolation system has been introduced. (LeukoLOCK Total RNA Isolation System, Ambion, Austin TX). This system allows the investigators to extract the white blood cells using a simple filter technique, inactivate the cells using a commercial hypertonic preservation agent (RNAlater, Ambion, Austin, TX), and keep the sample refrigerated for several days without degradation or alteration of the mRNA. This has greatly simplified initial processing, and has resulted in good enrollment to the study.
5. Data analysis has been in terms of the initial and 24-hour samples being compared with the 7-day (or discharge) samples. This has been a very useful way to analyze the data, since each patient is, basically, his or her own control. Alternatively, we have analyzed the data by using pooled 7-day samples as controls for the entire group. This has allowed inclusion of several patients who failed to return for the 7-day study. In future research in this area, we propose to obtain samples on a group of 10 normal volunteers, to act as a population control. This group will be selected from hospital and university personnel, from volunteers, and will be selected to match the age and

sex distribution of the trauma patients – i.e., age 20 to 40, 80% men. But this refinement is beyond the scope of the present study.

6. Analysis of the data shows several patterns very similar to the results seen in the rat model. The following table documents twenty genes, all of which appear to be elevated following injury. Of note, several of these are the human equivalents to the genes found to characterize the response to shock and injury in the rodent model.

<i>Gene</i>	<i>Protein and function</i>
ALOX5	Arachidonate 5-lipoxygenase (cytoplasmic enzyme)
B3GALT3	Membrane-bound galactotransferase enzyme
B3GNT1	Galactotransferase enzyme
CA4	Carbonic anhydrase 4; cell surface bound enzyme
CLEC4D	C-type lectin domain family 4; regulatory protein
CXCL5	Chemokine (C-X-C motif) ligand 5, chemokine, cytokine
F5	Coagulation factor, membrane receptor
GPR30	G-coupled membrane estrogen receptor
HSPA1B	Heat shock (70kDa) protein 1b
HSPA6	Heat shock (27kDa) associated protein 6
IL1RN	Interleukin 1 receptor antagonist
IMP3	Small nucleolar riboprotein
JAK2	Janus kinase 2; JAK-STAT pathway
PLAUR	Plasminogen activator; urokinase membrane receptor
PTGFR	Prostaglandin F receptor
SLC1A3	Glutamate aspartate membrane transporter
SOCS3	STAT-induced STAT inhibitor; cytokine regulatory protein
ST3GAL6	Membrane glycosyltransferase enzyme
THEDC1	S-acyl fatty acid synthase thioesterase
TNFSF13B	Tumor necrosis factor ligand (TNF superfamily); cytokine
TREML4	Immunoglobulin trans-membrane receptor

Research objective #4: Assess the response of patients to administration of modified resuscitation procedures. Deliverable: The effect of the experimental agents upon signature genes, cellular response, and cellular damage in patients.

1. All three potential agents, arginine, glutamine, and allopurinol, have shown improvement in survival in whole-animal models of shock, all can be given intravenously, and all are safe when given to humans in the doses contemplated. However, responding to guidance at the Trauma Portfolio Review (Product Line Review, San Antonio, TX, January 6, 2010), we have modified the study to employ only one of the three agents, specifically glutamine. It was felt by the review panel that trying to study all three agents initially would result in data which would be difficult to interpret, and would not be approved by the Human Research Protection Office (HRPO). Nor, it was noted, would such a study be useful for possible subsequent FDA submission. On the basis of work carried out so far, and after carefully reviewing the relevant literature, glutamine has been selected for human

use. It should be noted that none of these three agents have been used clinically in post-injury treatment before. However, glutamine has been used more extensively than either of the other two agents in critically ill patients, and largely for that reason was selected for use in the present study. Optimal dosage has already been established by other investigators, and it would seem reasonable to use the most-used agent for this novel application.

2. Our intent will be to evaluate the response of patients to glutamine as a component of resuscitation, using a prospective, randomized, double-blind model, and to study the response in terms of a panel of genes drawn from previous results in patients with injuries described above.
3. The study has been approved by the Institutional Review Board of the University of Missouri - Kansas City, and has been approved by the Human Research Protection Office (HRPO), Office of Research Protections (ORP), U.S. Army Medical Research and Materiel Command (USAMRMC). It has been re-submitted to the UMKC IRB for final approval prior to entering patients. This approval is anticipated to be granted by June 30, which would allow beginning the study on or shortly after July 1, 2010.
4. To complete this portion of the study, we are requesting a no-cost extension of the contract for 1 year. While no funding remains in the contract, this study can be supported by other funding within the Shock Trauma Center.

REPORTABLE OUTCOMES

PUBLICATIONS AND PRESENTATIONS (Italized items in previous reports)

1. Yang R, Tan X, Thomas AM, Shen J, Qureshi N, Morrison DC, Van Way CW 3rd. *Crocetin inhibits mRNA expression for tumor necrosis factor-alpha, interleukin-1-beta, and inducible nitric oxide synthase in hemorrhagic shock. JPEN J Parenter Enteral Nutr.* 2006 Jul-Aug;30(4):297-301.
2. Yang R, Tibbs BM, Chang B, Nguyen C, Woodall C, Steppacher R, Helling T, Van Way CW 3rd. *Effect of DHEA on the hemodynamic response to resuscitation in a porcine model of hemorrhagic shock. J Trauma.* 2006 Dec;61(6):1343-9.
3. Yang R, Van Way CW III, Tan X, Thomas A, Shen J, Steppacher R, Qureshi N, Morrison DC. *Alanine-glutamine dipeptide inhibits expression of inflammation-related genes in hemorrhagic shock. Clinical Nutrition Week of the American Society for Parenteral and Enteral Nutrition, Dallas. February 12-15, 2006. (Oral presentation). Clinical Nutrition week,. 2006, p145. (Abstract of Distinction)*
4. Thomas A, Yang R, Van Way CW III, Quresh N. *A Preliminary micro-array study of the response to hemorrhagic shock and to therapy with crocetin and alanine-glutamine dipeptide. Kansas City Area Life Sciences. March 30, 2006. Kansas City Life Sciences. 2006, p163. (Abstract)*
5. Yang R, Van Way CW III, Tan X, Thomas AM, Shen J, Steppacher R, Qureshi N, Morrison DC. *Alanine-glutamine dipeptide inhibits inflammatory mediator genes in hemorrhagic shock. Kansas City Area Life Sciences. March 30, 2006. Kansas City Life Sciences. 2006, p153. (Abstract)*
6. Yang R, Thomas A, Tan X, Landis M, Qureshi N, Morrison D, Van Way CW III. *Hemorrhagic shock in the rat: comparison of femoral and carotid techniques. Twenty-ninth Annual Conference on shock. Broomfield, Colorado. June 3-6, 2006. Shock. 25:S97,2006. (Abstract)*
7. Yang R, Yan X, Thomas AM, Steppacher R, Qureshi N, Morrison DC, Van Way CW 3rd. *Alanine-glutamine dipeptide inhibits expression of inflammation-related genes in hemorrhagic shock. JPEN J Parenter Enteral Nutr.* 2007 (Jan-Feb) 31(1):32-6.
8. Yang R, Martin L, Woodall C, Thomas AM, Qureshi N, Morrison DC, Van Way CW III. *Administration of glutamine following hemorrhagic shock restores cellular energy, reduces cell apoptosis and damage, and increases survival. JPEN J Parenter Enteral Nutr.* 31:94-100,2007.
9. Pontes-Arruda A, Van Way CW III. *Feeding During Sepsis: What Works, What Doesn't. Clinical Nutrition Week of American Society of Parenteral and Enteral Nutrition, Jan 27-31, 2007 (Phoenix, Arizona). (Presentation and abstract)*
10. Smit J, Kaltashov IA, Cotter RJ, Vinogradov E, Perry MB, Haider H, and Qureshi N.

Structure of a novel lipid A obtained from the lipopolysaccharide of Caulobacter crescentus. Innate Immunity Feb 14:25-36, 2008.

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CONCLUSION

This study has rested on two basic hypotheses. The first is that agents given during and (for a short period of time) after resuscitation can favorably influence the response to hemorrhagic shock and injury. The second is that studies of gene response studies, specifically microarray and RT-PCR studies, could be used to assess the response of patients being treated for injuries to such therapeutic agents.

To date, both hypotheses have been shown to be true in the animal model. Work continues to determine optimum therapeutic agents to be used during and after resuscitation. In addition, development has begun on a multiple-gene RT-PCR assay incorporating a group of signature genes which have been identified as uniquely responsive to shock and therapy. Development of this assay will greatly simplify future work in this area, both in our laboratories and in those of other investigators.

Studies are ongoing to validate these hypotheses in patients. During the next year, which will be a no-further-cost extension of the contract, we will subject the use of glutamine to preliminary clinical trial.

We anticipate final completion of the proposed research over the next year.

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1. Abstract for ESPEN 2010 (European Society for Clinical Nutrition)

Category: Epigenetic effects of nutrition

Title: The proteasome as a proposed locus for nutrient modulation of gene expression

Precis: To investigate mechanisms for the improvement of survival in hemorrhagic shock by the nutrients arginine and glutamine, a cell-culture based model of hypoxia was investigated. The proteasome, a proteolytic intracellular organelle, is central to regulatory mechanisms controlling the inflammatory reaction. It was found to react to hypoxia by altering both its proteolytic activity and the composition of its protein subunits. Constitutive subunits, α , β , and γ , were partially substituted by inducible subunits LMP7, LMP2, and LMP10. Gene expression and expression of the protein subunits themselves were altered by arginine levels in the culture medium. Modulation of the proteasome appears to represent a mechanism for nutritional modulation of the inflammatory response.

**Pre-Feeding With Omega 3 Fatty Acids Suppresses Inflammation
Following Hemorrhagic Shock**

Running head: Fish Oil Reduces Shock-Induced Inflammation

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1 **Pre'cis:** Inclusion of fish oil in the diet before induction of experimental hemorrhagic
2 shock in rodents reduced lung edema and liver IL-1• mRNA and increased HSP-25
3 mRNA.

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5 Foundation for Education and Research, the US Army Medical Research and Materials
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7 Foundation.

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9 American Society for Parenteral and Enteral Nutrition, Feb 10-13, 2008 Further data
10 was presented at the meeting of the International Society for the Study of Fats and
11 Lipids (ISSFAL), May 19-22, 2008.

12 **Keywords:** fish oil, hemorrhagic shock, heat shock protein 25, IL-1• , iNOS

1 **ABSTRACT** *Background:* Hemorrhagic shock followed by resuscitation stimulates an
2 inflammatory response. This study was designed to test the hypothesis that pre-feeding
3 experimental animals with fish oil rich in • -3 fatty acids (FAs) will attenuate that
4 response. *Methods:* Male Sprague-Dawley rats (350±30 g) were randomly assigned to
5 three groups: sham (n=12), control (n=24), and fish oil pre-fed (• -3, n=24). In the • -3
6 group, rat chow diet was supplemented with fish oil (600 mg/kg/day, 25% • -3 FA). The
7 control and sham groups were supplemented with corn oil instead of fish oil. Under
8 fluothane anesthesia, hemorrhagic shock was induced, with mean arterial pressure
9 maintained at 25-30 mmHg for 30 minutes. Resuscitation was carried out over 30
10 minutes by giving 21ml/kg lactated Ringer's solution and returning the shed blood. Half
11 of each group was sacrificed at 30 minutes and half at 4 hours post-resuscitation. Liver
12 samples were assayed for mRNA, for inducible nitric oxide synthase (iNOS) and
13 interleukin (IL)-1• as indicators of inflammation, and heat shock protein 25 (Hsp25) as a
14 chaperone protein. The lungs were weighed both wet and after dessication, and the
15 lung tissue edema index was evaluated. *Results:* Survival to 4 hours was 100% in all
16 groups. At 30 minutes post resuscitation, expression of mRNA for iNOS was
17 significantly elevated in the control group, but normal in the • -3 group. At 4 hours post-
18 resuscitation, expression of mRNA for Hsp25 was significantly increased in the • -3
19 group. IL-1• mRNA expression was significantly increased in the control group, but not
20 in the • -3 group. Lung edema index was significantly lower in the • -3 group than either
21 sham or LR groups. *Conclusions:* Fish oil pre-feeding in a rodent model of hemorrhagic
22 shock was associated with reduced liver mRNA expression of iNOS and IL-1• ,

Fish Oil Reduces Injuries in Hemorrhagic Shock

- 1 increased mRNA expression of Hsp25, and decreased lung edema. These findings
- 2 support the validity of the study hypothesis.

INTRODUCTION

Hemorrhagic shock followed by resuscitation is known to stimulate a systemic inflammatory response. Preventing the catastrophic consequences of hemorrhagic shock remains as an unsolved problem in clinical treatment of trauma. Previous studies from our group have shown that a number of inflammation-related genes are over-expressed in hepatic tissues following hemorrhagic shock and resuscitation (1-2) and these responses precede tissue apoptosis and resulting tissue damage.

The hypothesis in the present study was that pre-feeding experimental animals with fish oil rich in ω -3 fatty acids (FAs), specifically eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), would help to minimize the post-shock inflammatory responses. ω -3 FAs have been reported to modulate inflammation and have been used clinically for that purpose. (3,4) In this study, fish oil, rich in EPA and DHA, was pre-fed to animals who were then subjected to sub-lethal hemorrhagic shock. Control rats were fed with ω -6 FAs.

MATERIALS AND METHODS

Nutritional supplements and pre-feeding

Ten to twelve week old male Sprague-Dawley rats (350 \pm 30g, Charles River Laboratories, Wilmington, MA) were randomly assigned into sham group (n=12), control group (n=24), and ω -3 group (n=24) then fed with appropriate diets. Animals were maintained in the Animal Care Center of the University of Missouri-Kansas City, and were kept on 12:12-hour light-dark cycle with free access to food and water. In the ω -3 FA group, rat chow diet was supplemented with commercial fish oil 600 mg/kg/day (25% DHA and EPA). This provided omega-3 fatty acids equivalent to about 2% of the daily

energy requirement). In the sham and control groups, an equivalent amount of corn oil was added to the rat chow. Diets were stored in the dark at X°C until fed, animals were allowed access to food ad libitum and food remaining in the cage from the previous day was discarded. All of the diet preparations were done by the supplier (Dyets, Inc, Bethlehem, PA). Animals were subjected to hemorrhagic shock after four weeks of pre-feeding.

Hemorrhagic shock model

All rats were anesthetized with isoflurane (Baxter Healthcare Corporation, Deerfield, IL) using an anesthesia vaporizer (SurgiVet, Inc. Waukesha, WI) with 100% oxygen, while breathing spontaneously. Isoflurane was adjusted from 3% to 1% after induction and then maintained at 1% until the conclusion of the experiment. The femoral artery and femoral vein was cannulated through a short groin incision (1-2). Heparin (200 units/kg) was administered following cannulation. Hemorrhagic shock was induced by removing blood slowly through the left femoral arterial cannula. The mean arterial pressure (MAP) was maintained at 25-30 mmHg for 30 minutes in the control and • -3 FA groups with periodic withdrawal of additional blood. MAP was measured continuously using a blood pressure monitor (Micro-Med, Louisville, KY). Resuscitation was carried out by giving 21ml/kg LR and returning the shed blood, at 0.5 ml/min. The animals in the sham group received anesthesia and surgical preparation, and were then kept anesthetized for an equivalent length of time to the control and experimental groups, but were not subjected to shock. At the end of 30 minutes of shock, the cannulas were withdrawn and the artery and vein ligated. In half the animals, liver samples were collected for mRNA at 30 minutes post-resuscitation (n = 30; sham = 6, control = 12, and

• -3 = 12). The rest of the animals (n = 30) were awakened, and placed in a clean cage. They were re-anesthetized, and liver samples collected for mRNA at 4 hours post-resuscitation. All animals were sacrificed while anesthetized using aortic transaction.

Real-time PCR reaction

Liver tissue was collected as noted above, and gently homogenized. Isolation of mRNA was done using a commercial kit (QUIAGEN RNA/DNA Mini Kit, QUIAGEN Inc., Valencia, CA). The oligonucleotides used are shown in Table I. RT-PCR was carried out using the two step method. First, cDNA was synthesized using a commercial kit (SuperScript III First Strand Synthesis System, Invitrogen Corp., Carlsbad, CA). Quantitative PCR was then carried out using the Opticon 2 real-time PCR detection system (Bio-Rad Laboratories, Waltham, MA). Oligonucleotides were prepared as specified in Table I, and were ordered from a commercial supplier (Sigma-Genosys, Woodlands, TX). The resulting data were analyzed using the $2^{-\Delta\Delta C_T}$ approach to allow comparison among the three groups.

Fatty Acid analysis

To verify that the dietary changes had resulted in the incorporation of significant amounts of DHA and EPA into membrane lipids, FA analysis was carried out in red blood cells (RBCs) and in liver cells. The former were separated from the plasma by centrifugation of blood at 1500 x g for 20 min at 4°C. RBCs were stored at -70°C until analysis. Packed RBCs were thawed and 50 µl were dried at 45°C under nitrogen. Thereafter, the samples were directly methylated by the addition of 0.5 mL of 14% boron trifluoride methanol (Sigma, St. Louis, MO) and then heated at 100°C for 10 min. This generated fatty acid methyl esters (FAME) from the glycerophospholipids in the

1 RBC membranes.(5) After cooling, 1.0 ml water and 1.0 ml hexane containing 50 mg/L
2 butylated hydroxytoluene (BHT, an antioxidant, Sigma, St. Louis, MO) were added.
3 Following manual-shaking for 30 sec, the samples were centrifuged for 3 min at 1500 x
4 g at room temperature. The hexane layer was collected, the solvent was evaporated
5 45°C under nitrogen, and the FAME thus generated were reconstituted with 50 µL
6 hexane and analyzed by flame ionization gas chromatography (GC).
7 Liver samples were first homogenized, the cell pellet isolated by centrifugation, and
8 frozen until analyzed. Total liver lipids were extracted with methanol and methylene
9 chloride (both containing 50 mg/L BHT) as described. (6) The organic phase was
10 collected and the solvent evaporated under nitrogen in a 45°C water bath. The samples
11 were methylated as described. (7). The samples were analyzed by gas chromatography
12 (GC-14A, Shimadzu, Columbia, MD) using a fused silica capillary column (SP-2560,
13 100 m; Supelco, Bellefonte, PA). FAMES are reported as percent of total and were
14 identified by comparison with known standards.

15 *Cytokine analysis*

16 TNF• and IL-6 were analyzed using the Quantikine assay kit (R&D Systems,
17 Minneapolis, MN). This assay uses a quantitative sandwich enzyme immunoassay
18 technique (ELISA). The monoclonal antibody is pre-coated onto a microplate. After
19 placing the samples, controls, and standards into the wells, available mouse IL-6 is
20 bound by the antibody. Excess is washed, and a second enzyme-linked polyclonal
21 antibody was added. After a second wash, a solution of enzyme substrate was added.
22 This was then converted to a yellow chromogen with an acidic stop solution, and samples

were measured spectrophotometrically. Actual amounts were calculated from a standard curve.

Nitrite analysis

Nitric oxide activity was estimated by measuring nitrite, using a Greiss reagent system (Promega, Madison, WI). Samples, standards, and controls were placed in a 96-well plate. Sulfanilamide solution (1% sulfanilamide in 5% phosphoric acid) was added. After a 10-minute incubation, N-1-naphthylethylenediamine dihydrochloride (NED) was added. After another 10 minutes, the absorbance 520-550 nm was measured on a plate reader. Concentrations of nitrite were read from the standard curve.

Lung tissue edema index

After removal, the lungs were patted dry with a tissue and weighed. They were then dried overnight in an oven and re-weighed. The lung edema index was calculated as the ratio between the wet weight and the dry weight.

Institutional approval

All animal care and experimental procedures were carried out according to the Guidelines of the Laboratory Animal Center of the University of Missouri-Kansas City, which is a facility accredited by the American Association for the Accreditation of Laboratory Animal Care. The experimental protocols under which this work was carried out were approved by the Institutional Animal Care and Use Committee of the University of Missouri-Kansas City.

Statistical analysis

Group sizes (n=12) were determined using power analysis (PASS, NCSS, Kaysville, UT). A group size of 12 was determined to provide an 80% chance of detecting a 2:1

variation in the levels of cytokines, or a 2-fold increase in the mRNA measured by RT-PCR using the 2^{-ΔΔCt} method. Data analysis was carried out with Student's t-test for experimental versus control groups, using Microsoft Excel (Microsoft, Redmond, OR), and NCSS (NCSS, Kaysville, UT). Analyses including the sham group were carried out using one-way analysis of variance (NCSS, Kaysville, UT). Data results are presented as means ± SEM. A significance level of 0.05 was used for all analyses.

RESULTS

Shock Preparation.

All animals survived shock and resuscitation in all groups. Among the groups, there was no difference in the return of blood pressure to baseline following shock and recussitation. Except for the sham group, all animals were maintained at 25-30 mm Hg for 30 minutes by periodic withdrawal of blood, without having to add blood back. There were no detectable differences among the groups in terms of weight, cannulation time, or amount of blood withdrawn (except again for the sham group) (Table II).

Lipid Analysis

Levels of DHA and EPA were found to be significantly elevated in membrane lipid preparations as compared with controls in both tissue types, while levels of arachidonic acid (AA) were correspondingly reduced (Tables III and IV). Thus, we concluded that the four week time period selected for these studies was sufficient for incorporation of significant amounts of omega-3 fatty acids into lipid membranes.

TNF•, IL-1•

TNF• was measured 30 minutes post-shock and recussitation, since it is known to be characteristic of an early response to hemorrhagic shock. As expected, it was

markedly elevated in both control and fish oil groups, but not in the sham group. As the variability among animals was relatively large, this precluded our ability to detect significant differences between the two shock groups. IL-6 was measured in the group of animals at 4 hours post recussitation, since it is characteristically a later response. Interestingly, levels of this cytokine were elevated in sham animals as well as both shock groups, with no significant detectable difference among the three groups. (Table V)

Nitric oxide production (Table V)

The nitrite concentrations, reflective of the presence of inducible nitric oxide synthase, were elevated at 30 minutes in all three groups, and the concentrations were reduced to about one-quarter of peak values at 4 hours. Once again, there were no significant detectable differences among the groups.

Lung edema index (Table V)

The lung edema index was measured in tissue samples collected from the animals at 4 hours. It was slightly higher for the control than for the sham, but not significantly so. However, it was significantly lower in the fish oil group than either sham or control groups (which were fed the same diets).

mRNA for IL-1 α , IL-6, IL-8, IL-10, Hsp25 (Table VI)

The levels of mRNA for cytokines was measured in hepatic tissues at 4 hours. Expression of mRNA for IL-1 α was increased in the control group, but not in the α -3 group, as compared with the sham group. The levels of expression of mRNA for IL-6 and IL-8 were reduced in both shock groups as compared with sham-treated animals, but there was no significant difference between the shock groups. At 4 hours post-

1 resuscitation, expression of mRNA for Hsp25 was increased in the • -3 group relative to
2 the control diet-fed group of rats.

3 *iNOS mRNA*

4 The levels of mRNA for iNOS were measured at both 30 minutes and 4 hours. At
5 30 minutes, the control shock group showed a significant elevation in iNOS message as
6 compared with the sham and fish oil fed groups of rats. At 4 hours, there was no
7 significant difference among groups, but variability was sufficiently high that meaningful
8 differences were not obtainable.

10 **DISCUSSION**

11 Dietary supplementation with omega-3 fatty acids has been well established as a
12 daily dietary supplement for reduction in risk of cardiovascular disease, and is used
13 experimentally for alleviation of certain acute systemic inflammatory diseases such as
14 asthma, chronic obstructive pulmonary disease, and inflammatory bowel disease. (8)
15 There have been a number of trials with omega-3 fatty acids in critically ill patients. In a
16 meta-analysis in 2001 22 published reports were reviewed. The analysis indicated that
17 the overall effect of fish oil in immunomodulation was to reduce complications, but not
18 mortality. (9) On the other hand, a smaller meta-analysis published in 2008 of 3 reports
19 in the literature on the use of EPA and DHA in the acute respiratory distress syndrome
20 (ARDS) showed a strong beneficial effect on mortality and other outcome measures.
21 (10) Clinical use of omega 3 fatty acids in the systemic inflammatory response
22 syndrome, however, remains an open question, and in part prompted the present study.

23 A number of investigators have examined the potential protective contribution of

1 fish oil in experimental shock. Ertel, et al, (11) pre-fed mice with omega-3 fatty acids for
2 3 weeks, subjected them to hemorrhagic shock and recussitation, and harvested
3 peritoneal macrophages and splenocytes at 24 hours post recussitation. They found
4 that fish oil prevented an increase in levels of circulating prostaglandin E-2 (PGE2)
5 release seen in mice fed with corn oil or safflower oil. Dietary fish oil also prevented the
6 reduction in antigen presentation capacity and consequent suppression of interleukin 1
7 (IL1) release. Our own studies reported here were carried out with liver cells at 4
8 hours, and have provided evidence that pre-feeding with fish oil suppressed early
9 mRNA expression only for several components of the inflammatory response. This
10 suggests either that the time course may be important or that liver cells may react
11 differently from macrophages.

12 Additional studies in this area have been done using sepsis models. Oz, et al, for
13 example, showed that 6 days of a fish oil diet could protect rats from the hepatic
14 damage associated with lipopolysaccharide (LPS) administration.(12) A modest
15 reduction in circulating levels of interferon-gamma was observed at 4 hours after LPS
16 administration, but no change was seen in either IL-6 or TNF- α .

17 Babcock, et al, studied LPS-stimulated murine macrophage-like RAW 264.7 cells,
18 and showed that levels of phosphorylation of proteins in the mitogen-activated protein
19 kinase (MAPK) pathway were significantly reduced. The reduction was associated with
20 suppression of activation of the transcription factor activator protein-1 (AP-1). (13)
21 Novak, et al, also studied RAW 264.7 cells stimulated with LPS, and found that
22 pretreatment with omega-3 fatty acids inhibited I κ B phosphorylation and decreased
23 NF- κ B activation. There was a consequent decrease in levels of TNF- α mRNA and

protein. (14) Lo et al showed a similar finding for pre-feeding with EPA alone. (15)

Mishra et al studied murine aortic endothelial cells, showing that “oxidized EPA” inhibited endothelial MCP-1 and IL-8 expression, and that it inhibited NF- κ B activation but not AP-1 activation. No specific oxidation product was identified. (16) Other studies provided evidence that oxidized EPA is a strong activator of PPAR α , and the above effects were not seen in PPAR α deficient cells. Auto-oxidation of EPA occurs naturally, and the authors speculated that the oxidized compounds have anti-inflammatory properties acting through a PPAR α dependent mechanism. (16)

As the data from the present study indicate, one of the constant results of feeding with omega-3 fatty acids is incorporation of EPA and DHA into cell membranes. Is it possible that the changed hydrophobic local lipid environment of the cell membrane affects receptor function on the cell surface? There is evidence, for example, that DHA and EPA can affect the characteristics of crucial areas of the cell membrane. A review by Ma, et al, summarizes work done in several cell lines on the effect of omega-3 fatty acids on lipid rafts.(17), substructures of the lipid membrane, sometimes characterized as detergent-resistant membranes. The available evidence would strongly support the conclusion that these structures are critically important in cell signaling, concentrating many of the receptors and signaling proteins. Switzer, et al, from the same group, studied the effect of omega-3 fatty acids on T-cell membrane function, and hypothesized that these fatty acids modulate T-cell function by altering composition of the lipid rafts. (18) Schley, et al, found that EPA and DHA altered the composition of lipid rafts in human breast cancer cells, specifically decreasing epidermal growth factor receptor (EGFR) activity and increasing phosphorylation of EGFR and mitogen-activated protein

kinase (MAPK).(19) Chen, et al, showed that DHA was incorporated into the lipid rafts of human vascular endothelial (hRVE) cells, and displaced cholesterol. Further, this was associated with inhibition of TNF- α and IL-1 α induced cellular adhesion molecule (CAM) expression and with TNF- α induced NF- κ B activation.(20)

The "classic" explanation for the effects of omega-3 fatty acids on regulation of inflammatory host responses is that the administration of omega-3 fatty acids promotes the formation of eicosanoids, which are anti-inflammatory, rather than pro-inflammatory.

(21) Ingestion of fish oil (EPA and DHA) leads to decreased prostaglandin E₂, thromboxane A₂, and leukotriene B₄, with increases in thromboxane A₃, prostacyclin PGI₃, and leukotriene B₅. More generally, the leukotrienes, prostacyclins, thromboxanes, and prostaglandins derived from omega-3 fatty acids are less pro-inflammatory than the corresponding compounds derived from omega-6 fatty acids. There are, however, powerful anti-inflammatory metabolites from the omega-6 fatty acid arachidonic acid, and thus the "omega-6 are pro-inflammatory and omega-3 are not" is oversimplified. (22) Nevertheless, the present study was not designed to address such mechanisms in the modulation of the post-recussitation response to shock.

The results from this study do, however, support our hypothesis that pre-feeding with omega-3 fatty acids can alter the inflammatory response to hemorrhagic shock. Pre-feeding with a diet rich in omega-3 fatty acids, as compared with a diet rich in omega-6 fatty acids, clearly modulated the host response as assessed by IL-1 α , iNOS, and HSP-25 following hemorrhagic shock. Elevation of Hsp-25 following a stress is associated with increased cytoprotection. Lung edema, an index of the systemic inflammatory reaction, was decreased. It seems clear from the above discussion that

1 elucidating the mechanism(s) responsible for this effect will be an important next step in
2 this area of investigation.

3

4 **CONCLUSIONS**

5 Fish oil pre-feeding in a rodent model of hemorrhagic shock was associated with
6 reduced liver mRNA expression of iNOS and IL-1•, increased mRNA expression of
7 Hsp25, and decreased lung edema. These findings support the hypothesis that
8 supplementation with DHA and EPA can modulate the inflammatory reaction.

9

10

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11. **Table 1: Oligonucleotide Primers**

Protein	Direction	Primer Sequence
IL-1•	Forward	5'- GGATGATGACGACCTGC - 3'
	Reverse	5'- CTTGTTCCGTTATGTTCTG - 3'
iNOS	Forward	5' - GGAGAGATTTTTACGACACCC - 3'
	Reverse	5' - CCATGCATAATTTGGACTTGCA - 3'
Hsp25	Forward	5' - TGTCAGAGATCCGACAGACG - 3'
	Reverse	5' - GCCTTCCTTGGTCTTCACTG - 3'
IL-6	Forward	5' - CAAAGCCAGAGTCCATTCAGAGC - 3'
	Reverse	5' - GGTCTTAGCCACTCCTTCTGT - 3'
IL-8	Forward	5' – GACTGTTGTGGCCCGTGAG – 3'
	Reverse	5' – CCGTCAAGCTCTGGATGTTCT – 3'
IL-10	Forward	5' – AAAGCAAGGCAGTGGAGCAG – 3'
	Reverse	5' TCAAACCTCATTCATGGCCTTGT – 3'
• -actin	Forward	5' - TTGCTGACAGGATCGAGAAG - 3'
	Reverse	5' - TAGAGCCACCAATCCACACA - 3'

1
2 Table II: Hemorrhagic shock parameters (mean \pm SEM)
3

	Sham	Control	• -3
Weight (grams)	341 \pm 4	349 \pm 6	347 \pm 6
Cannulating time (minutes)	7.5 \pm 0.4	8.2 \pm 0.3	8.1 \pm 0.4
Blood removed (mL)	0	9.2 \pm 0.3	9.6 \pm 0.2

4
5

1 Table III: Lipid content of red blood cell membranes

2

RBC EPA, DHA and Arachidonic Acid

Group	EPA (C20:5n3)		DHA (C22:6n3)		AA (C20:4n6)	
	mean	SD	mean	SD	mean	SD
Control (n=24)	0.19	0.13	1.91	0.41	21.09	2.37
Fish Oil (n=24)	1.82	0.27	4.88**	0.94	16.94	1.46
p value	<0.01		<0.01		<0.01	

3

4

5 Table IV: Lipid content of cell membranes extracted from liver homogenate

6

Liver EPA, DHA and Arachidonic Acid

Group	EPA (C20:5n3)		DHA (C22:6n3)		AA (C20:4n6)	
	mean	SD	mean	SD	mean	SD
Control (n=24)	0.05	0.01	4.18	2.15	21.08	3.40
Fish Oil (n=24)	2.31	0.67	13.04	2.25	13.95	2.63
p value	<0.01		<0.01		<0.01	

7

8

1 Table V: Results of cytokine (pg/mL plasma) and lung edema index (wet / dry weight)

Quantity measured	30 minutes post shock			4 hours post shock		
	Sham	Shock-Control	Shock-n3	Sham	Shock-Control	Shock-n3
TNF- α	<1	1220 \pm 445	1605 \pm 657	--	--	--
IL-6	--	--	--	589 \pm 26	568 \pm 15	567 \pm 13
Nitric Oxide	190 \pm 11	166 \pm 18	189 \pm 20	42.6 \pm 1.3	42.6 \pm 1.2	44.8 \pm 1.9
Lung edema index	--	--	--	5.14 \pm 0.18	5.28 \pm 0.28	4.69 \pm 0.14***
All data expressed as mean \pm SEM						
***p<.05 (t test, Shock-omega 3 vs Shock-control)						

2

3

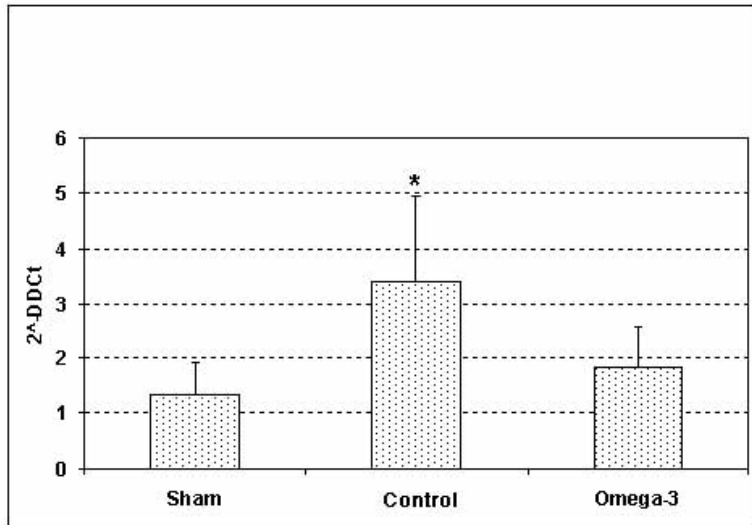
1 Table VI: Results of RT-PCR analysis for cytokines, Hsp25, 4 hours post-shock

	4 hours post shock		
Quantity measured	Sham	Shock-Control	Shock-fish oil
IL-1 α mRNA †	1.34 \pm 0.56	3.39 \pm 1.56***	1.83 \pm 0.75***
IL-6 mRNA †	1.36 \pm 0.92	.22 \pm 0.08	.36 \pm 0.11
IL-8 mRNA †	1.17 \pm 0.61	0.48 \pm 0.12	0.53 \pm 0.28
IL-10 mRNA †	1.05 \pm 0.32	2.82 \pm 1.20	2.18 \pm 0.82
iNOS mRNA †		6.39 \pm 3.97	12.3 \pm 9.37
Hsp25 mRNA †	1.36 \pm 0.29	2.11 \pm 0.74***	1.14 \pm 0.22***
†Relative to α -actin, calculated using 2 ^{-DDCt} method			
All data expressed as mean \pm SEM.			
***p<.05 (t test, shock omega-3 vs shock-control)			

2

3 Table VII Results of RT-PCR analysis for iNOS

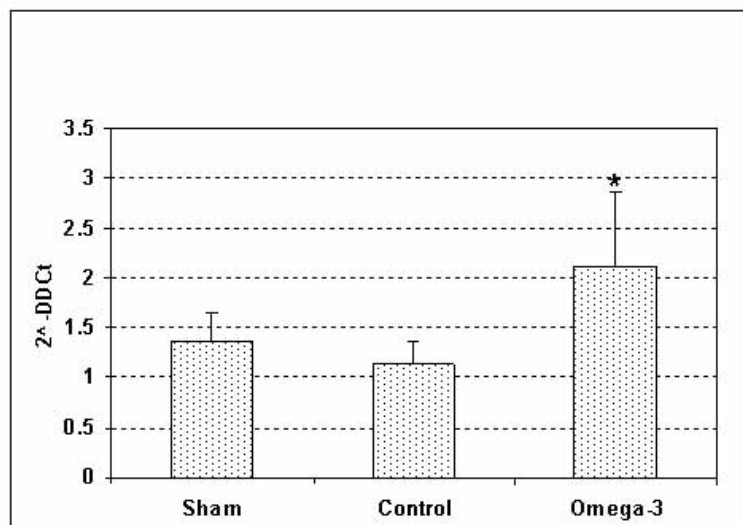
Quantity measured	Time	Sham	Shock-Control	Shock-fish oil
iNOS mRNA	30 minutes	1.84 \pm 0.72	6.76 \pm 2.19***	1.57 \pm 0.73***
iNOS mRNA	4 hours	1.05 \pm 0.32	6.39 \pm 3.97	12.29 \pm 9.37
†Relative to α -actin, calculated using 2 ^{-DDCt} method				
All data expressed as mean \pm SEM.				
***p<.05 (t test, shock-fish oil vs shock-control)				



1

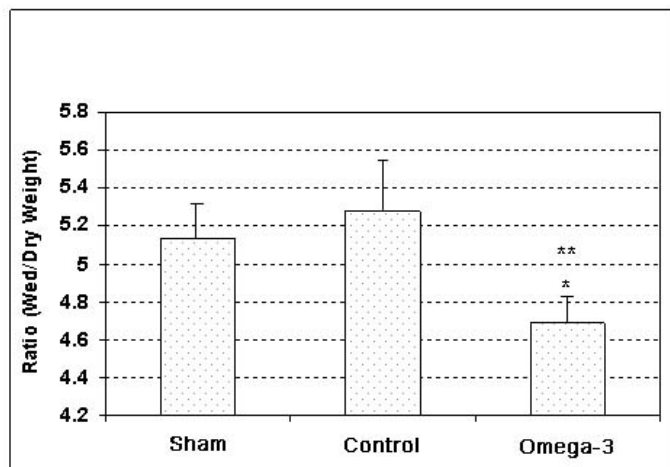
2 Figure 1. Relative expression of mRNA for interleukin-1 beta (IL-1beta), showing that the group
3 which was fed omega-3 fatty acids had lowered expression of this inflammatory gene.

4



1

2 Figure 2. Relative expression of mRNA for Heat Shock Protein 25 (Hsp25), showing that this
3 chaperone protein was expressed more strongly in the group fed omega-3 fatty acids than in the
4 group fed omega-6 lipids (LR).



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2 Figure 3: Lung edema index, showing less water accumulation in the group receiving omega-3
3 fatty acids than in either the sham group or the group fed omega-6 lipids (LR).

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Appendix 3. Abstract for American Society of Parenteral and Enteral Nutrition, February 8-12, 2010.

TITLE: GENE ACTIVATION IN WHITE BLOOD CELLS FOLLOWING ARGININE ADMINISTRATION IN A MODEL OF HEMORRHAGIC SHOCK

ABSTRACT BODY:

Introduction: The addition of arginine to a standard resuscitation regimen was found in a previous experimental study to increase survival following lethal hemorrhagic shock. The present study tested the hypothesis that the use of arginine would alter gene activation as sampled in circulating white cells.

Methods: A sublethal model of shock was used. A total of 14 Sprague-Dawley rats (300 gm) were divided into 4 groups: sham, shock alone, shock resuscitated with Ringer's lactate, and shock resuscitated with supplemental arginine. Animals were anesthetized with fluothane. The femoral artery and vein were cannulated. After stabilization, blood was withdrawn, and a mean blood pressure of 25 mm Hg was maintained for 30 minutes (except for the sham group). In the two resuscitation groups, 28 ml/kg Ringer's lactate was given over 30 minutes. In the arginine group, arginine 300 mg/kg was added to the resuscitation solution. Blood for analysis was drawn at baseline, 30 minutes, and 4 hours. The white cells were isolated, RNA extracted, and microarray analysis was carried out using the Affymetrix system, using 31,099 probes. Data analysis was done with canonical pathway analysis (Ingenuity Systems, Inc).

Results: Significant gene activation within 26 separate canonical pathways was seen in shock resuscitated with Ringer's lactate. Supplemental use of arginine was associated with upregulation of genes in 5 pathways: hepatic fibrosis/stellate cell activation, PPAR/RXR activation, androgen/estrogen metabolism, TGF-beta signaling, and IL-10 signaling. Arginine produced decreased activation in 6 pathways: hepatic cholestasis, FXR/RXR activation, PPAR activation, acute phase response signaling, synaptic long term potentiation, and xenobiotic metabolism signaling. Analysis of the changes within pathways indicate that arginine did not change the overall pattern of gene activation, but that it affected a number of signalling elements within the involved pathways. Arginine had highly selective effects. For example, while IL-1 was up-regulated in both resuscitation groups, IL-1 and IL-1 receptor accessory protein were further activated in the arginine group. MAP-kinase 11 was de-activated, while MAPK9 was further activated. Significantly, the gene SOCS2 (suppressor of cytokine signalling 2), was more activated following arginine as compared with Ringer's lactate alone.

Conclusions: Administration of arginine in this model differentially affected 11 canonical pathways. The pattern suggests that its effects on survival is associated with a broad range of effects at the gene activation level on both signaling and metabolic pathways.

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